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New Interpretation of a Nudibranch Central Nervous System Based on Ultrastructural Analysis of Neurodevelopment in *Melibe leonina*. I. Cerebral and Visceral Loop Ganglia

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Abstract. Development of the 'cerebropleural' ganglia in the dendronotid nudibranch *Melibe leonina* (Gould, 1852) was examined by electron microscopy of semi-serial sections through larval stages. Although comparative neuroanatomical studies suggest that the paired cerebropleurals of nudibranchs are formed by fusion of cerebral and pleural ganglia, plus all other ancestral ganglia of the visceral loop, my study indicates that the pleural ganglia are not part of these compound ganglionic masses. In *Melibe* larvae, the cerebral, optic, and rhinophoral ganglia, arise from pre-trochal cephalopedal ectoderm. At hatching stage, the visceral loop extends from the two cerebral ganglia, is non-ganglionated, and forms a complete circuit beneath the esophagus. Ganglia that subsequently develop along the visceral loop, which were identified as subintestinal, visceral, supraintestinal, and possibly right parietal ganglia, arise from placodes of visceropallial ectoderm that show torsional displacements. In addition, a cluster of neurons, presumed to be osphradial, lies close to the rim of the right mantle fold. Detorsion of the visceral loop is accomplished by migration of subintestinal neurons along the visceral loop fiber tract, not by visceral loop shortening. Localized elongation of a different segment of this fiber tract during metamorphosis displaces the visceral ganglion to the left, where it fuses with subintestinal and left cerebral ganglia.

Introduction

More than a century of comparative neuroanatomical studies on opisthobranchs have revealed a wide range of ganglionic fusions and cephalization within this gastropod group (Russell, 1929; Hoffmann, 1936; others reviewed by Bullock, 1965; Schmekel, 1985). In species showing evidence of fusions, homologous ganglionic regions have been inferred by extrapolation from the layout of distinct ganglia, connectives, and peripheral nerves found in presumably more primitive species. Although these interpretations have been largely accepted by most contemporary gastropod systematists and neurophysiologists, they are nevertheless conjectural (see Dorsett, 1986). This is particularly true for nudibranchs, in which ganglia of the central nervous system (CNS) show extreme consolidation. Ambiguity about homologous ganglionic regions reduces the taxonomic value of neuroanatomical characters and contributes to the uncertainty about phyletic origins and relationships of the opisthobranchs (see Minichev, 1970; Minichev and Starobogatov, 1978; Gosliner, 1981, 1991; Gosliner and Ghiselin, 1984; Haszprunar, 1985b, Schmekel, 1985). Furthermore, possible misconceptions about ganglionic fusion patterns can confound comparative neurobiological studies—a lamentable situation for an animal group that is otherwise very amenable to neuroethological investigation (see Kandel, 1979; Willows, 1985–1986).

Studies of gangliogenesis in prosobranch gastropods indicate that the CNS typically develops from a similar groundplan, with various derived conditions arising later in ontogeny [compare studies of Crofts (1937) and Moritz (1939) with those of Honegger (1974) and Demian and Yousif (1975)]. This groundplan, in which discrete ganglia are interconnected in a specific pattern, is not greatly altered during the subsequent development of some prosobranchs, and it appears also in Gosliner's (1981) proposal for the ancestral opisthobranch nervous system.

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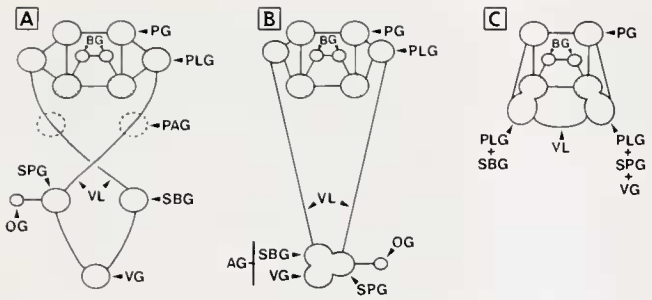


Figure 1. Opisthobranch central nervous systems showing varying degrees of cephalization, ganglionic fusions, and euthyneury. Dorsal views; cerebral ganglia stippled. A. Possible ancestral condition (adapted from Gosliner, 1981); distinct parietal ganglia found in some extant opisthobranchs. B. *Aplysia californica* (adapted from Kriegstein, 1979a, b). C. aeolid nudibranch (adapted from Russell, 1929); note visceral loop emerging from ganglionic mass projecting posteriorly from cerebral ganglia. AG = abdominal ganglion; BG = buccal ganglia; OG = osphradial ganglion = PG = pedal ganglion; PAG = parietal ganglion; PLG = pleural ganglion; SBG = subintestinal ganglion; SPG = supraintestinal ganglion; VG = visceral ganglion; VL = visceral loop.

Reasoning from studies of a variety of opisthobranchs, Gosliner (1981) suggested that the common ancestor of this group had paired cerebral, pleural, and pedal ganglia interconnected around the esophagus, a long streptoneurous visceral loop (twisted due to torsion) punctuated by subintestinal, supraintestinal, and visceral ganglia, and an osphradial ganglion connected to the supraintestinal ganglion (Fig. 1A). Extant opisthobranchs also have a pair of buccal ganglia, and some species have an extra ganglionic pair, called the parietals, located anterior to the intestinals. Developmental studies on the anaspidean *Aplysia californica* have shown that all ganglia of Gosliner's ancestral opisthobranch appear during early larval development, but the visceral and intestinal ganglia fuse eventually to form what is called the abdominal ganglion (Fig. 1B) (Kriegstein, 1977a, b; Schacher *et al.*, 1979a, b). These results suggest that the true pattern of ganglionic fusions among other opisthobranchs might be revealed by studies of neurodevelopment.

The adult CNS of nudibranchs, which shows only three pairs of distinct ganglia surrounding the esophagus, is much more consolidated than that of aplysiids. The ganglia have been identified traditionally as the pedals, buccals, and cerebropleurals, with the latter incorporating the cerebral and pleural ganglia plus all other ganglia of the visceral loop (Fig. 1C). Developmental studies of nudibranchs have indeed shown that the cerebropleurals are constructed ontogenetically from precursor ganglia, but there are three different interpretations for the location and identity of these precursor ganglia (Thompson, 1958; Tardy, 1970, 1974; Bickell and Chia, 1979; Bickell and Kempf, 1983; Kempf *et al.*, 1987). Much of this confusion may stem from limited resolution provided by histological sections.

To address the controversy regarding the ontogeny of the cerebral and visceral loop ganglia in nudibranchs, I have cut semi-serial, ultrathin sections through sequential larval stages of the dendronotid nudibranch *Melibe leonina*. A review of the genus has been given recently by Gosliner (1987). General features of larval and metamorphic development in this species were described from histological sections by Bickell (now Page) and Kempf (1983). The interpretation of gangliogenesis given in the present paper and the following companion paper (Page, 1992) differs from that described in the earlier study.

Materials and Methods

Adults of *Melibe leonina* and their egg masses were collected from Patricia Bay, Vancouver Island, Canada.

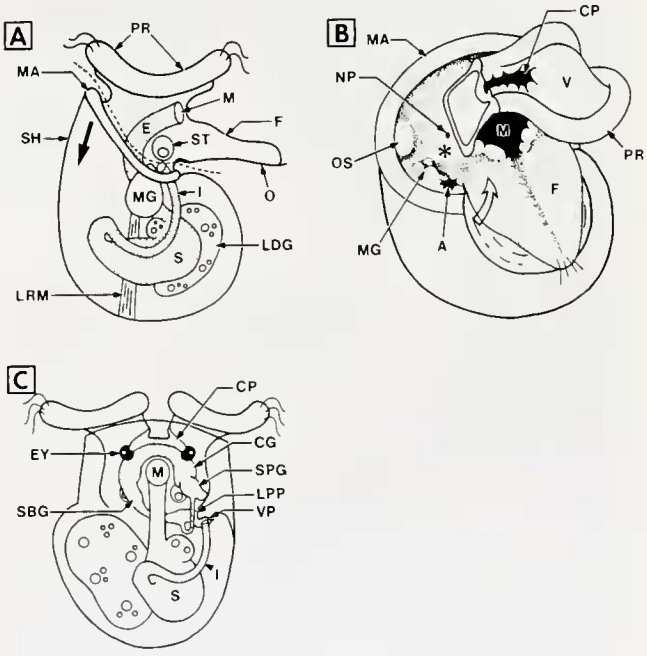


Figure 2. Veliger larvae of *Melibe leonina*. A. Lateral view, prior to mantle retraction, showing basic anatomy; gut is stippled. Broken line passes along floor of mantle cavity and demarcates cephalopedal mass from visceropallial mass. Arrow indicates displacement of mantle at mantle retraction. B. Oblique, antero-ventral view of young veliger; right velar lobe cut away to reveal mantle fold lining right mantle cavity. Swellings in mantle fold are osphradial neurons (OS) and apices of mantle gland (MG). The asterisk marks position of right pallial placode; open arrow indicates a site *beneath* the foot, where the left pallial placode is located. Note invagination of left cephalic plate (CP) within pre-trochal ectoderm. C. Dorsal view of veliger shortly after mantle retraction showing positions of cerebral ganglia and components of visceral loop (developing CNS stippled). Mantle gland omitted for clarity. A = anus; CG = cerebral ganglion; CP = cephalic plate; EY = eye; E = esophagus; F = foot; I = intestine; LDG = left digestive gland; LPP = left pallial placode; LRM = larval retractor muscle; M = mouth; MA = shell-secreting cells of mantle; MG = mantle gland; NP = nephrocyst pore; O = operculum; PR = prototroch (cilia not shown in 2B); S = stomach; SBG = subintestinal ganglion; SH = shell; SPG = supraintestinal ganglion; ST = statocyst; V = velar lobe; VP = visceral placode.

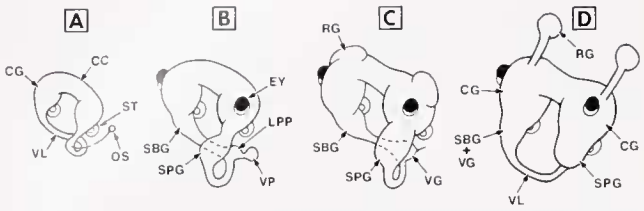


Figure 3. Summary sketches of developing ganglia of cephalic plate and visceral loop in *Melibe leonina;* postero-lateral views from right side. A. newly hatched larva. B. larva at mantle retraction stage. C. late larval stage. D. metamorphic stage. CC = cerebral commissure; CG = cerebral ganglion; EY = eye; LPP = left pallial placode; OS = osphradial neurons; RG = rhinophoral ganglion; SBG = subintestinal ganglion; SPG = supraintestinal ganglion; ST = statocyst; VG = visceral ganglion; VL = visceral loop; VP = visceral placode.

Larvae that hatched from egg masses laid in the field or laboratory were reared according to the method of Bickell and Kempf (1983). Under laboratory conditions and a rearing temperature of 12 to 14°C, larvae required a minimum of 5 weeks to complete pre-metamorphic development.

Larvae were anaesthetized as described by Bickell and Kempf (1983), and were fixed according to the method of Bickell and Chia (1979).

The area of the larval body containing developing ganglia was thin sectioned in whole or part with a diamond knife, and batches of eight to ten sections were collected on uncoated copper grids (150 mesh size). The grids were first washed in acetone and distilled water, then passed briefly through the flame of an alcohol burner; this eases the pick-up of floating sections, possibly by reducing the hydrophobicity of the grid surface. To discourage the tendency of sections to float towards the grid periphery, grids were bent slightly so that sections were lifted onto a convex grid surface without excess water. The central areas of wet sections were teased over openings between grid bars with an eyebrow hair mounted on an orange stick. With this method, two to six whole sections per grid could be viewed, with each gridload of sections representing approximately 0.8 μm of tissue thickness. Sections were stained for 90 min in aqueous 2% uranyl acetate and 8 min in 0.2% lead citrate at room temperature.

Initially, a general picture of the CNS within each specimen was reconstructed by photographing one section per two to three grids at a magnification of 873× on the negative with a Philips EM300. This gave a panoramic view of the section but still allowed resolution of small fiber tracts. Subsequently, areas showing important structural features were photographed at higher magnification from these and intervening grids.

The following larval stages were thin sectioned: newly hatched, 6 days old, just prior to mantle retraction (larval shell at full size but mantle fold not yet retracted), onset of mantle retraction, complete mantle retraction, hypertrophy of retracted mantle fold, and late stage larva with ceratal rudiments. Characteristics of the mantle fold were used to stage larvae because these can be recognized before sectioning (Bickell and Kempf, 1983). Stages fixed immediately after shell loss, and at 5 and 10 h after shell loss, were cut into serial 1 μm thick sections and stained with methylene blue and Azure II in sodium borax (Richardson *et al.,* 1960). All larval stages were also thick sectioned to corroborate gross features seen in ultrathin sections.

Results

General

Planktotrophic veliger larvae of *Melibe leonina* consist of two major parts: a cephalopedal mass, which includes the ciliated velar lobes, distal esophagus, and foot; and a visceropallial mass, which consists of the remainder of the gut encased by the mantle (pallium) (Fig. 2A). The mantle is derived from the embryonic shell gland and consists of a squamous epithelium lining the inner wall of the shell and a peripheral rim of large cells that secrete larval shell material. Epithelium extending from the shell-secreting cells to the cephalopodium is called the mantle fold, although it is unclear if this is also derived from the shell gland. In newly hatched *Melibe* larvae, the mantle fold is only a few cells wide and thus the mantle cavity it

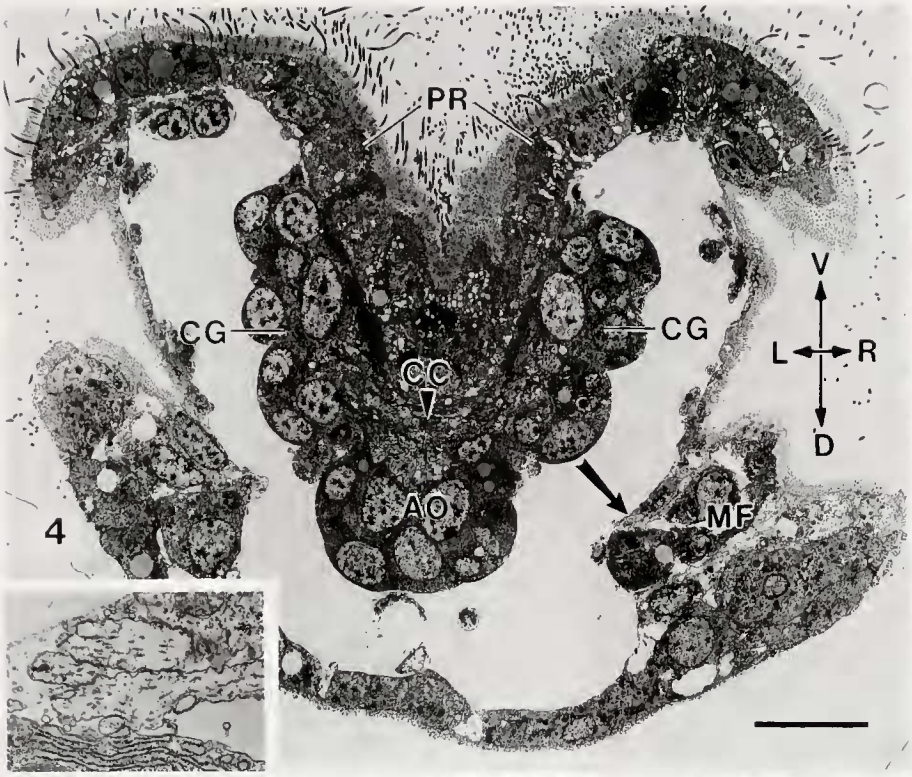
Figure 4. Cross section through newly hatched larva passing through cerebral ganglia (CG), cerebral commissure (CC), and cephalic apical organ (AO). The prototroch (PR) is indented over the mouth. Arrow indicates position of osphradial nerve (enlarged in inset) within the right mantle fold (MF). Orientation arrows: D = dorsal; V = ventral; L = left; R = right. Scale, 10 μm.

Figure 5. Cross section through newly hatched larva showing part of cerebral commissure (CC) extending into left cerebral ganglion (CG). Arrow indicates cilia within apical organ cell. Scale, 2 μm.

Figure 6. Cross section through apex of 6-day-old larva showing cephalic plates (CP) invaginated from pre-trochal ectoderm. PR = prototroch. Scale, 10 μm.

Figure 7. Frontal section through left side of larva at onset of mantle retraction showing multilayered, invaginated cephalic plate (CP) overlying cerebral ganglion (CG). Arrowhead indicates mitotic cell; arrow indicates ingressing cephalic plate cells. V = velar lobe. Scale, 5 μm.

Figure 8. Frontal section through right side of larva at onset of mantle retraction showing eye (EY) overlying dorsal area of cerebral ganglion (CG). CC = cerebral commissure. Scale, 5 μm.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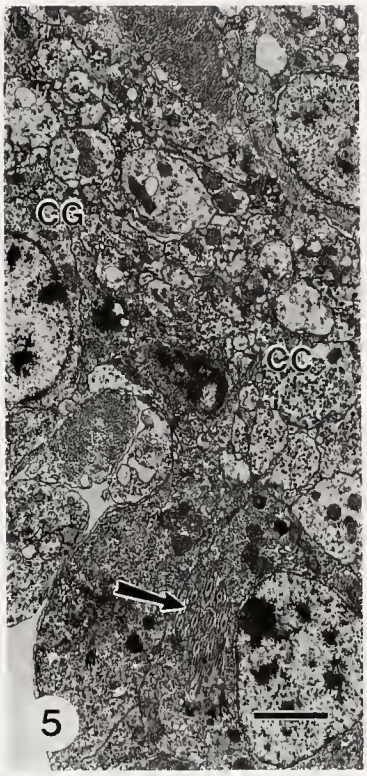
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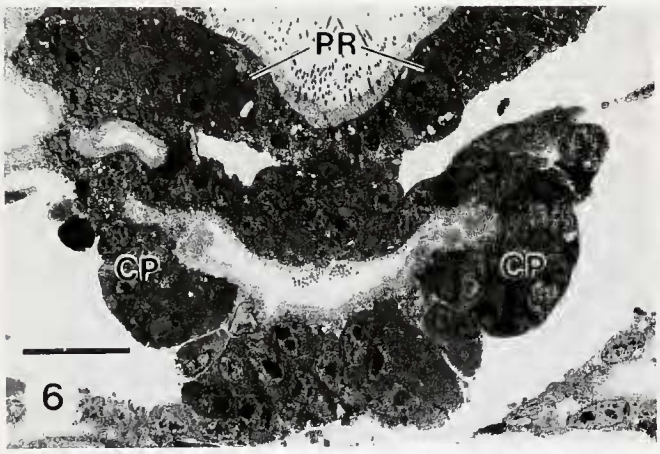
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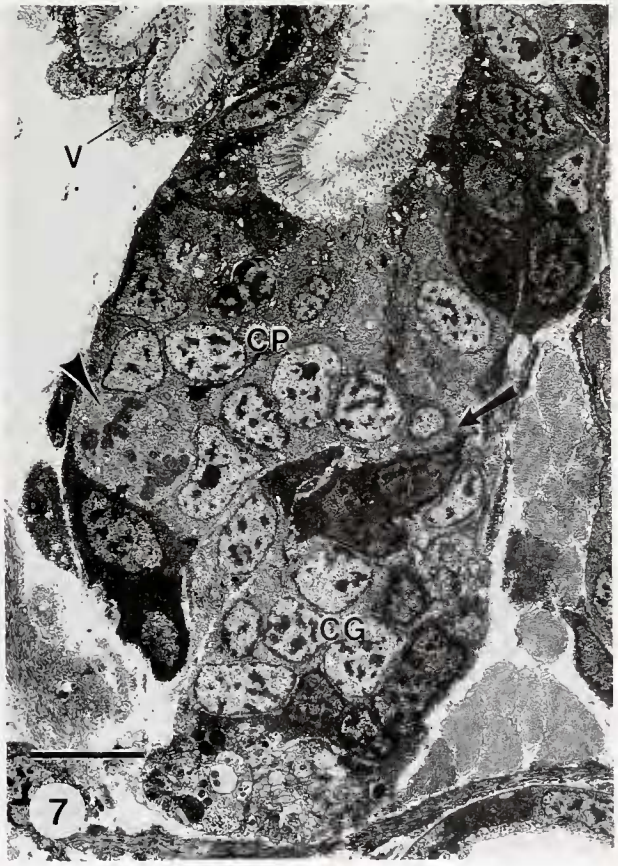
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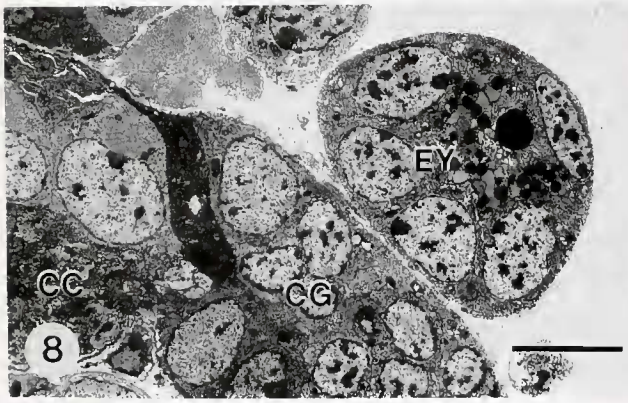
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defines is extremely shallow. The anus opens into the mantle cavity on the right ventro-lateral side, indicating slightly less than 90° torsion of the gut (Fig. 2B).

The shell growth that occurs during the first half of larval life is accompanied by a deepening of the right side of the mantle cavity, which extends from the anus over to the dorsal side (Fig. 2B). The left mantle cavity, which extends from the anus along the ventral aspect of the larva, remains shallow. It is important to note that the right mantle fold in partially torted *Melibe* larvae is equivalent to the left mantle fold of gastropods showing full 180° of torsion. It is this side that retains the various components of the pallial complex (ctenidium, osphradium, kidney) in monotocardian prosobranchs.

Midway through the larval phase of *Melibe,* shell secretion is arrested and the mantle fold detaches from the shell aperture and retracts posteriorly. The future post-metamorphic dorsum and cerata are formed from retracted mantle fold.

Two days after metamorphic shell loss, the basic characteristics of the adult nervous system are evident. The post-metamorphic CNS is formed by consolidation of many ganglionic primordia that arise during larval development by cellular ingression from thickened placodes of ectoderm. Neurogenic ectodermal cells often show mitotic figures.

To identify ganglionic primordia in *Melibe,* the locations of their respective neurogenic ectodermal placodes were compared to those that give rise to specific ganglia in prosobranchs. For these comparisons, it is important to distinguish three main areas of larval ectoderm, as illustrated in Figure 2. Ectoderm of the cephalopedal mass is subdivided into pre- and post-trochal areas by the band of large velar ciliated cells (the prototroch) that runs around the periphery of the velar lobes (Fig. 2B). The distinction between cephalopedal and visceropallial ectoderm is less distinct, but the latter shows torsional displacement whereas cephalopedal ectoderm does not. The pores of the left and right nephrocysts (large terminal cells of protonephridia) provide a convenient marker for the posterior limit of cephalopedal ectoderm (Fig. 2B). The nephrocysts do not exhibit torsional displacement in young larvae, although the left member is pulled further posteriorly than the right during mantle retraction. The anus and mantle gland (previously called larval kidney cell) lie posterior to the nephrocysts and show evidence of torsion (Figs. 2A, B).

The development and post-metamorphic fate of ganglia derived from pre-trochal cephalopedal ectoderm and from ectodermal placodes within the visceropallium are described below. Results are summarized by the diagrams in Figures 2C and 3. The companion paper (Page, 1992) describes ganglionic derivatives of post-trochal cephalopedal ectoderm.

Pre-trochal cephalopedal ganglia

Two placodes of neurogenic ectoderm, called cephalic plates, flank the mid-sagittal plane of the pre-trochal cephalopedal zone (Fig. 2B). Cells ingressing from the cephalic plates form the paired cerebral ganglia, eyes, optic ganglia, and rhinophoral ganglia.

In newly hatched veligers, each cerebral ganglion consists of neuronal cells clustered around a neuropile that is continuous with the fiber tract of the cerebral commissure. In cross sections, the cerebral ganglia and commissure form a horseshoe shaped complex (Fig. 4), perched above the distal esophagus, that parallels the trajectory of the prototroch where it arches above the mouth. The peculiar cells of the cephalic apical organ, which have an internal vacuole containing many cilia, are located on the dorsal side of the cerebral commissure (Fig. 5).

The paired cephalic plate placodes that overlie the cerebral ganglia are invaginated only slightly at hatching, but 6 days later the invaginations have deepened and are connected by a trough (Fig. 6). By the time of mantle retraction, the two invaginated cephalic plate lobes are composed of multiple layers of cells that sit as caps overhanging the ventro-lateral borders of the enlarging cerebral ganglia (Fig. 7). These cells ingress individually or in small clusters to the cerebral ganglia. Evidence of ingression is most prominent along the medial side of each invaginated cephalic plate lobe (Fig. 7).

Two eyes, each with a spherical lens and pigment granules, appear on the dorso-lateral surface of each cerebral ganglion prior to mantle retraction (Fig. 8). The eyes form adjacent to protuberances of the cerebral ganglia, which become the optic ganglia. The rhinophoral ganglia, which also arise from the cephalic plate, appear as mounds of cells on the cerebral ganglia of late stage larvae. During

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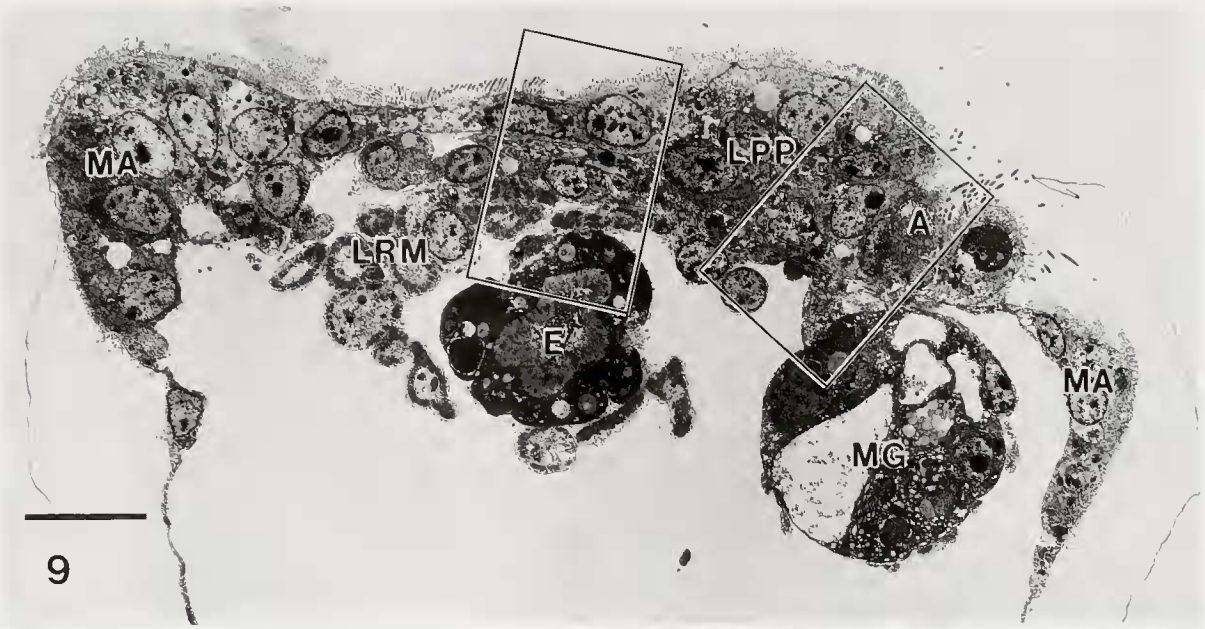
Figure 9. Cross section through newly hatched larva at a level just beneath the foot showing where the visceral loop completes its subesophageal trajectory. Area within left box enlarged in Figure 10; area within right box enlarged in Figure 11. A = anus; E = esophagus; LPP = left pallial placode; LRM = larval retractor muscle; MA = shell-secreting mantle cells; MG = mantle gland. Scale, 10 μm.

Figure 10. Left limb of visceral loop (arrows) extending towards right side beneath esophagus (E). Scale, 2 μm.

Figure 11. Right limb of visceral loop (R) leaving mantle gland (MG) to merge with left limb (L). A = anus. Scale, 2 μm.



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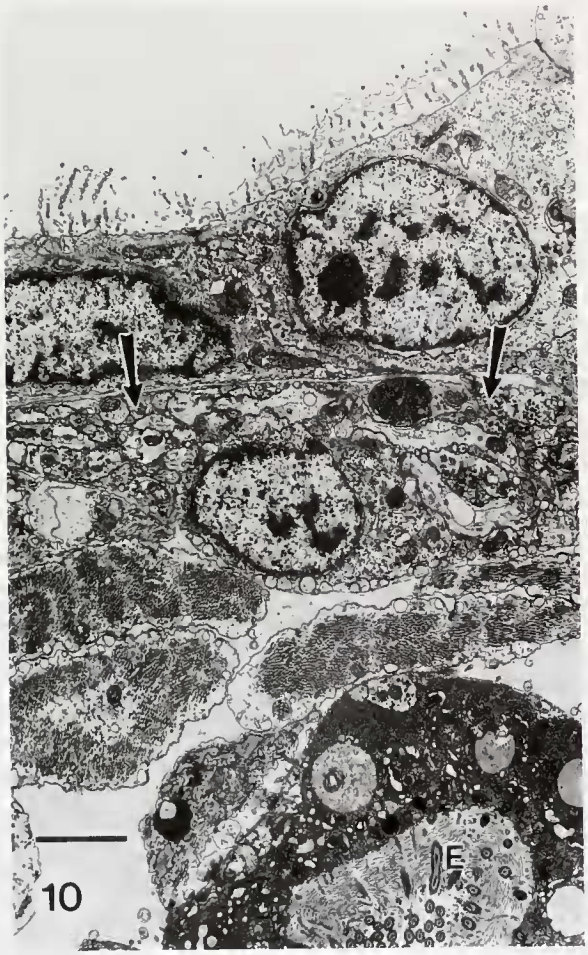

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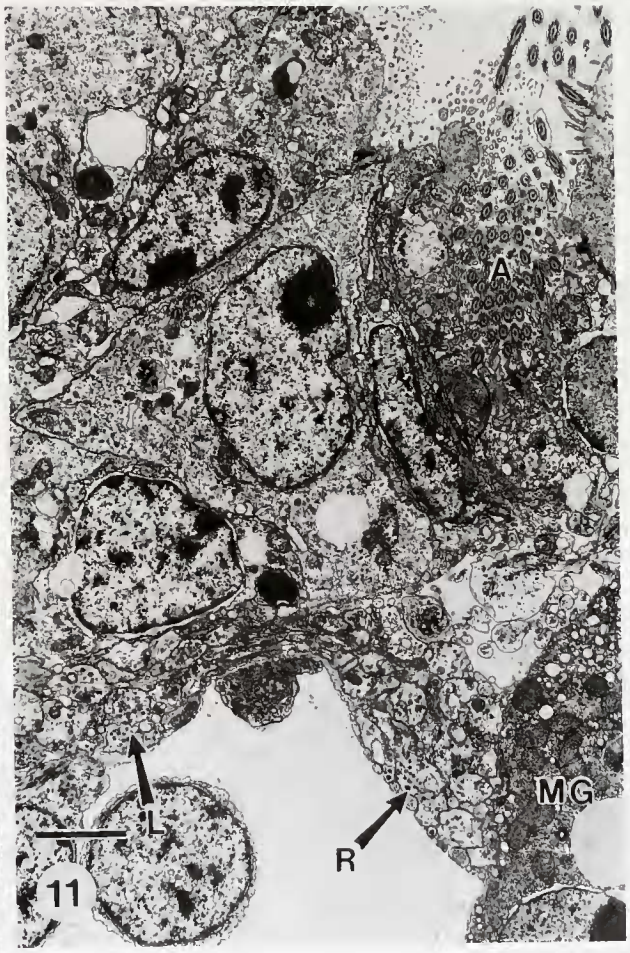

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metamorphosis, the rhinophoral ganglia are carried distally within the expanding oral hood (Bickell and Kempf, 1983).

Visceral loop ganglia

To minimize confusion, I will define my nomenclature for the visceral loop ganglia because varying schemes have been used by past authors. I use the terminology of Bullock (1965), in which the most anterior ganglia of the typical gastropod visceral loop are called left and right pleurals (Fig. 1A). These are followed by the sub- and supraintestinals and finally the unpaired visceral ganglion. The supraintestinal ganglion gives rise to a nerve extending to the osphradial ganglion. A second osphradial ganglion linked to the subintestinal ganglion is a characteristic restricted to diotocardian prosobranchs. In some pulmonates and opisthobranchs, but not in prosobranchs, extra ganglionic swellings are located anterior to the intestinal ganglia; these are called parietal ganglia. The visceral loop is long and twisted (streptoneurous) in many prosobranchs and a few opisthobranchs, but is short or untwisted (euthyneurous) in most extant opisthobranchs.

Sections through newly hatched larvae of *Melibe* show that the left and right limbs of the visceral loop fiber tract emerge directly from the neuropiles of their respective cerebral ganglia. The left limb swings beneath the esophagus to merge with the right limb at a point just medial to the anus (Figs. 9–11). The visceral loop is complete but nonganglionated at this initial larval stage and consists of at least 20 axons.

Figure 12 is a cross section through the left side of a newly hatched veliger, close to where the visceral loop on that side emerges from the cerebral ganglion. The section gives the impression that post-trochal ectodermal cells are ingressing from the left mantle fold toward the base of the cerebral ganglion. However, higher magnification reveals myofilaments within these subepidermal cells (Fig. 13); they are not ingressing pleural or parietal neurons. Differentiating subepidermal muscle cells are present in the same location on the right side of the larva. Indeed, the nuclear regions of many other fully and partially differentiated muscle cells are concentrated within the cephalopedal area of young larvae; these cannot be distinguished from neuronal elements in histological sections.

Right limb of visceral loop and osphradial neurons

After travelling a short distance posteriorly from the right cerebral ganglion, the right limb of the visceral loop in newly hatched veligers associates with a placode of thickened ectoderm that I call the right pallial placode (Fig. 14). The right pallial placode lines the deepest part of the right mantle cavity, immediately beneath the pore of the ipsilateral nephrocyst (Fig. 2B). At least one neuron originating from the right pallial placode is fully differentiated at hatching stage and is filled with many vesicles (Fig. 14). From this point, a small nerve extends anteriorly between the epithelial cells and underlying basal lamina of the mantle fold (Figs. 4 inset, 15). At least some of the axons forming this nerve arise from a cluster of sparsely ciliated neurons located close to the antero-lateral periphery of the right mantle fold. These neurons are associated with a mucous cell and their location corresponds to that of the osphradium and osphradial ganglion in prosobranch larvae (Figs. 2B, 15, 16). The fate of these osphradial neurons after mantle retraction requires further study. I failed to find them in sections of larvae fixed after onset of mantle retraction.

At least some of the neurons ingressing from the right pallial placode must be homologues of supraintestinal neurons, because they form a ganglion at the intersection of the visceral loop and osphradial nerve. However, the series of sections shown in Figures 17–20 reveal that this ganglion receives neurons from two ingression sites within the right pallial placode. These two sites may be sources of right parietal and supraintestinal neurons. However, an alternative interpretation is given in the discussion, and until this issue is clarified I will refer to the ganglion arising from the right pallial placode as the supraintestinal ganglion.

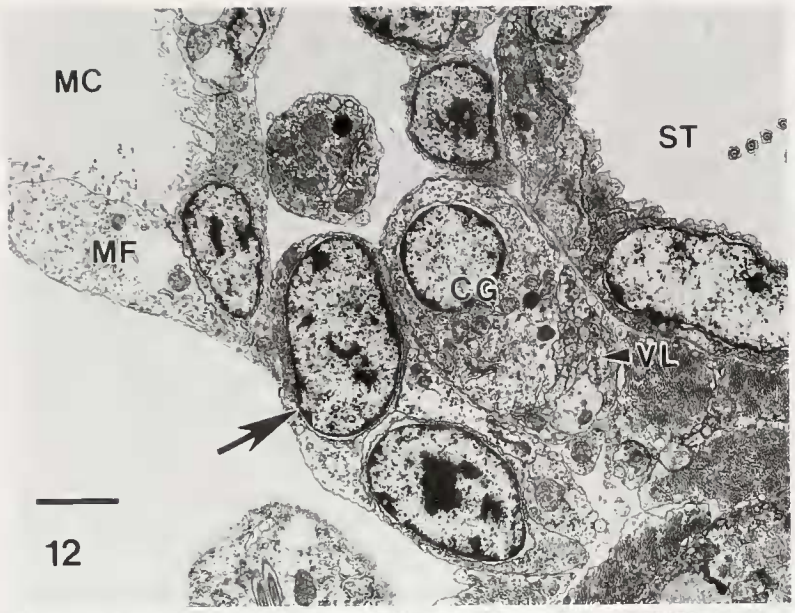
Figure 12. Cross section through left side of newly hatched larva close to where visceral loop (VL) emerges from cerebral ganglion (CG). Arrow indicates subepidermal cell (enlarged in Fig. 13) lying between mantle fold ectoderm (MF) and cerebral ganglion. MC = mantle cavity; ST = statocyst. Scale, 2 μm.

Figure 13. Detail from Figure 12 showing myofilaments (arrows) within subepidermal cell. Scale, 0.5 μm.

Figure 14. Cross section through newly hatched larva showing right limb of visceral loop (VL) deflecting towards right pallial placode (RPP). Asterisk marks neuron with many vesicles. Scale, 1 μm.

Figure 15. Mantle fold ectoderm on right side of 6-day-old larva showing differentiating osphradial neuron (asterisk) adjacent to axons of osphradial nerve (arrowheads). Arrow indicates vesicles that are enlarged in the inset. Scale, 1 μm; inset, 0.3 μm.

Figure 16. Frontal section through larva just prior to mantle retraction showing osphradial neurons (OS) and associated mucous cell (arrow) beneath shell-secreting periphery of mantle (MA). Supraintestinal ganglion (SPG) enlarged in Figure 19. H = hemocoel; N = nephrocyst; RPP = right pallial placode. Scale, 10 μm.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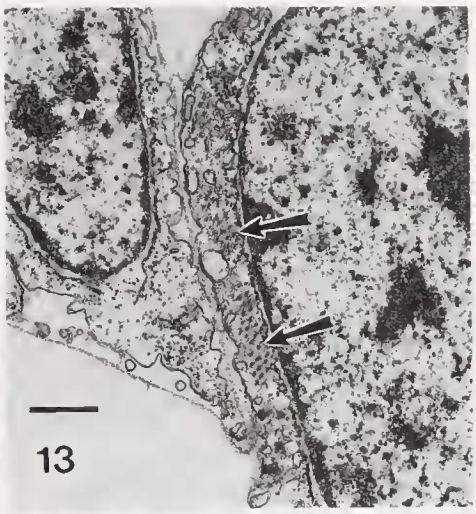
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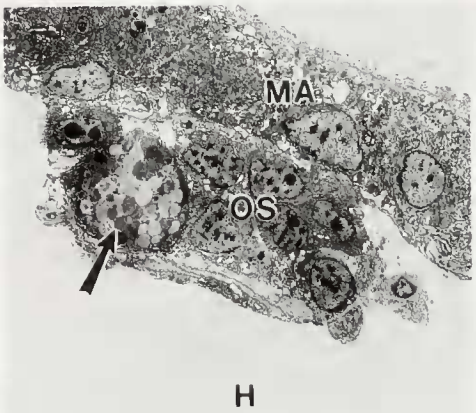
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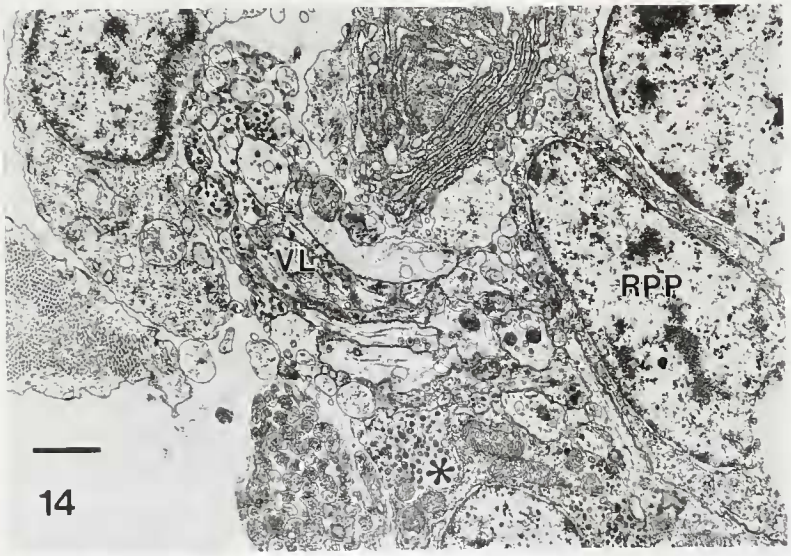
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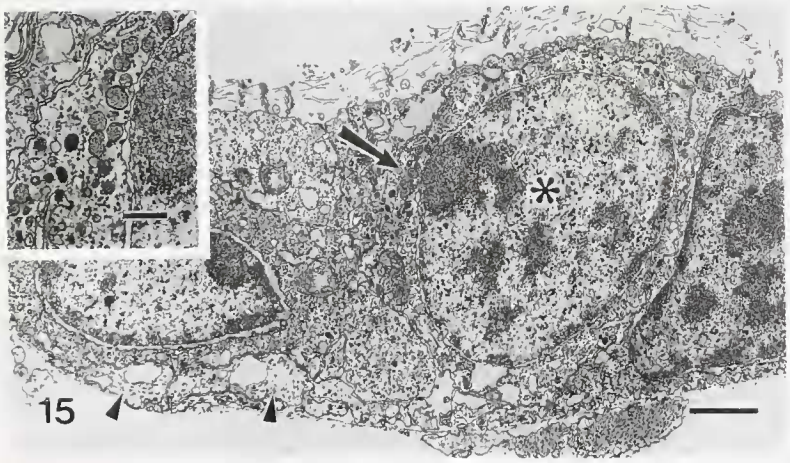
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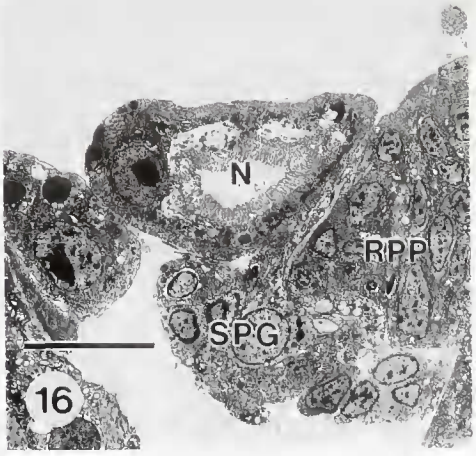
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Immediately after leaving the supraintestinal ganglion, the right limb of the visceral loop passes to the very large mantle gland that bulges into the hemocoel from the right mantle fold ectoderm (Fig. 20). This structure has been called the larval kidney complex, but evidence of an excretory role is weak and its ultrastructure is more consistent with a secretory function. The largest cell of the mantle gland, along with several associated secretory cells and the muscle fibers that invest the complex, are innervated by axons extending from the intimately associated visceral loop fiber tract.

In young larvae, the right limb of the visceral loop travels ventrally along the mantle gland until merging with the left limb (Fig. 11). However, the visceral loop lifts away from the mantle gland when the latter is displaced posteriorly during mantle retraction (Fig. 21). Although the peripheral rim of the mantle fold is pulled a great distance posteriorly during mantle retraction, the right pallial placode continues to be closely associated with the supraintestinal ganglion (Fig. 22). The ganglion lies lateral to the esophagus but projects dorsally.

Left limb of visceral loop and visceral ganglion

At the hatching stage, mantle ectoderm to the left of the anus is thickened by presumptive neurons. I call this the left pallial placode, because it is part of the left mantle fold. However, due to torsion, the placode is located toward the right side of the ventral aspect of the larval body (Figs. 2B, 9, 10). During later development, the left pallial placode is the only recognizable source of neurons along the entire left limb of the visceral loop in all larval stages examined. Furthermore, the only axons that leave this portion of the visceral loop extend to larval muscles, not to the ectoderm. Nevertheless, during subsequent development, cells are distributed along the entire left limb of visceral loop with a concentration appearing where the visceral loop emerges from the left cerebral ganglion (Fig. 23). These cells appear to be subintestinal neurons that originated from the left pallial placode on the right, ventrolateral side of the body and that migrated along the subesophageal trajectory of the visceral loop toward the left cerebral ganglion. Unlike the supraintestinal ganglion, which projects dorsally from the right cerebral ganglion (Fig. 22), the concentration of subintestinal neurons projects ventrally from the left cerebral ganglion (Fig. 23).

I could find no morphological evidence of a distinct ingression site for left parietal neurons.

The visceral placode, which is neurogenic visceropallial ectoderm for neurons of the future visceral ganglion, becomes recognizable at 6 days after shell loss. The visceral and left pallial placodes are contiguous. Figures 24–27, which is a series of frontal sections through a larva at onset of mantle retraction, show the positions of these two placodes and their relationship to the visceral loop. Note that the right limb of the visceral loop extends to the left pallial placode, not to the visceral placode.

During later development, the left limb of the visceral loop becomes progressively denuded of cells, presumably because most subintestinal neurons have migrated toward the left cerebral ganglion. Nevertheless, a small clump of neurons is apparent at the junction of the left and right limbs of the visceral loop and from this point, a prominent visceral nerve extends into the base of the visceral placode (Figs. 28–30). Ingressing visceral neurons do not form a ganglion immediately beneath the visceral placode. My observations suggest that visceral neurons migrate along the visceral nerve to form a consolidated ganglion on the visceral loop.

By the end of the larval phase, there are three neuronal concentrations along the visceral loop fiber tract: the supraintestinal ganglion behind the right cerebral ganglion, the subintestinal ganglion behind the left cerebral ganglion, and the visceral ganglion immediately adjacent to the anus (Fig. 3C). At this stage, the two intestinals have begun to fuse with their respective cerebral ganglia, but the visceral ganglion remains separate. Histological sections through metamorphic stages show that the visceral ganglion is relocated to the left side during the hours after shell loss, where it fuses with the left cerebral and subintestinal ganglionic mass (Figs. 3D; 31–33).

Discussion

It has been known since the last century that molluscan neurons are ectodermal derivatives (see reviews by Raven,

Figures 17–20. Series of frontal sections through right pallial placode (RPP) and supraintestinal ganglion (SPG) of a larva just prior to mantle retraction. N = nephrocyst.

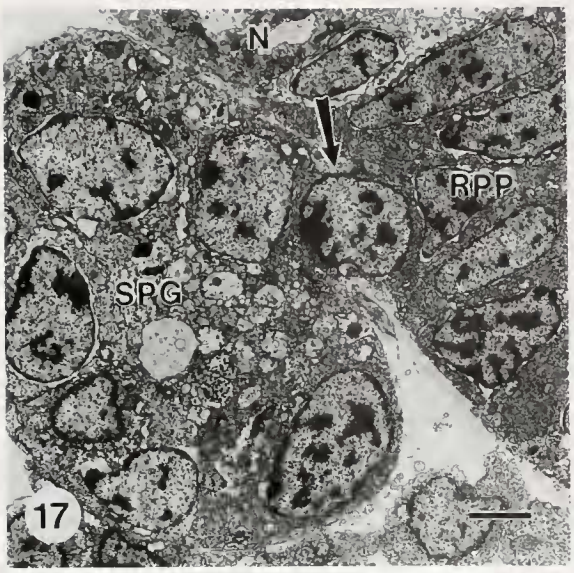
Figure 17. Arrow indicates presumptive neurons ingressing from right pallial placode to underlying ganglion. Scale, 2 μm.

Figure 18. Slightly deeper section showing no neuronal ingression. Scale, 2 μm.

Figure 19. Second site of neuronal ingression (arrow) coinciding with emergence of osphradial nerve (arrowheads) from the visceral loop (VL). Scale, 2 μm.

Figure 20. Visceral loop (VL) travelling from supraintestinal ganglion to mantle gland (MG). Scale, 2 μm.

Figure 21. Longitudinal section through supraintestinal ganglion (SPG) at full mantle retraction stage showing visceral loop (arrow) dissassociated from mantle gland (MG). Scale, 5 μm.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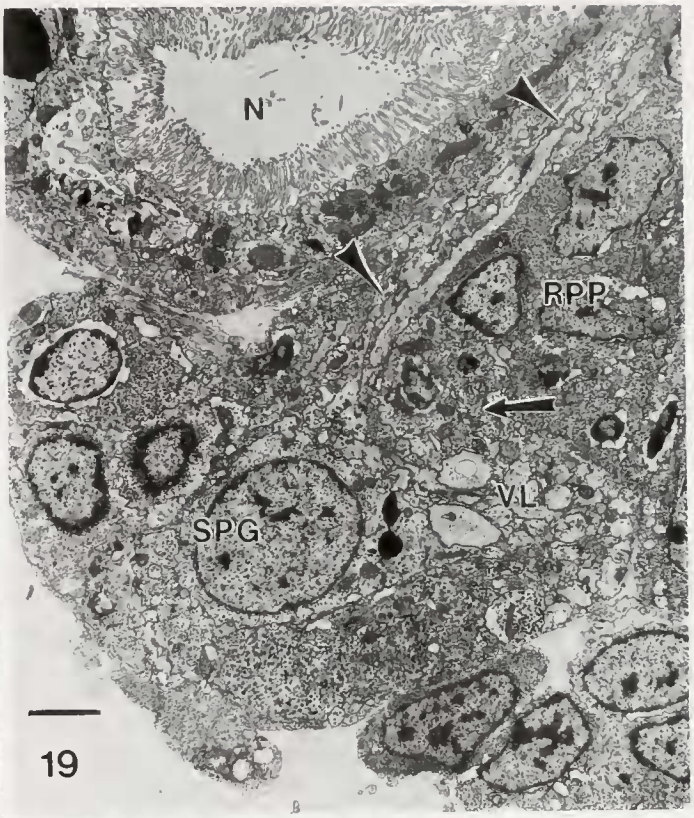
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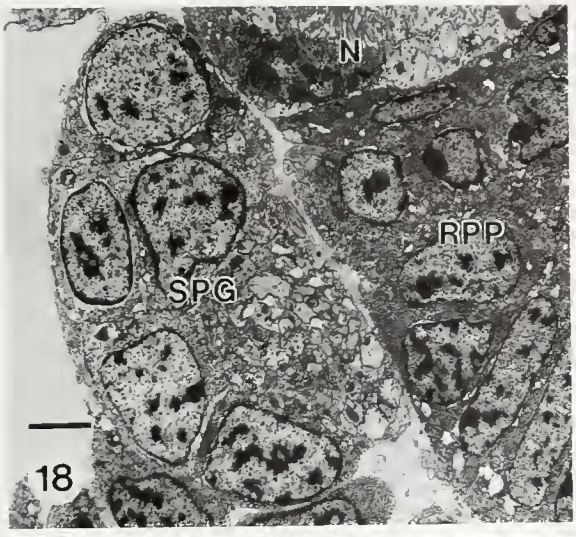
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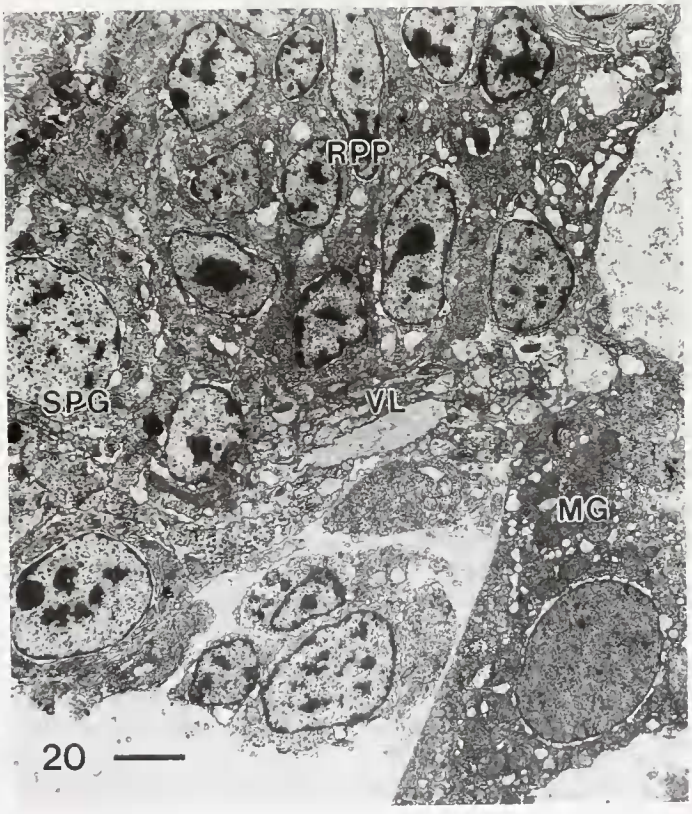
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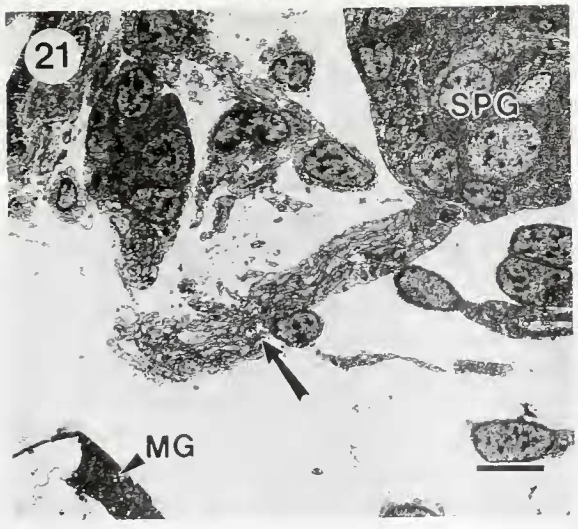
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1958; Moor, 1983). Furthermore, comparison of various histological accounts of prosobranch neurogenesis indicate that primordia of each ganglionic type arise from stereotypic locations within the ectoderm of the veliger (Smith, 1935; Crofts, 1937; Moritz, 1939; Creek, 1951; Régondaud, 1961, 1964; D'Asaro, 1969; Cumin, 1972; Guyomarc'H-Cousin, 1974; Honegger, 1974; Demian and Yousif, 1975). Cerebral ganglia always arise from the intravelar cephalic plates and intestinal, visceral, and osphradial ganglia arise from characteristic areas of visceropallial ectoderm. This phenomenon is particularly unambiguous in those gastropods that retain separate central ganglia through metamorphosis. Nevertheless, these sites appear to be a highly conserved feature of early gastropod neurogenesis, regardless of the final, species-specific form of the adult CNS (illustrated well by studies of Honegger, 1974; Demian and Yousif, 1975). Therefore, it is appropriate to identify ganglionic primordia in *Melibe* by comparing their ectodermal ingression sites to those described for the ganglia of other gastropods, particularly prosobranchs. Adjustments for variable degrees of torsional displacement among these species are made possible by the position of the anus relative to the cephalopedal mass.

Using *in situ* hybridization and immunofluorescence techniques, McAllister *et al.* (1983) found that mRNA for egg laying hormone (ELH) and related peptides in the opisthobranch *Aplysia californica* is located in neurosecretory bag cells located adjacent to the abdominal (intestinovisceral) ganglion, and also in a small number of central ganglia neurons. In veligers, cells containing this mRNA are "distributed throughout the entire length of the inner surface of the body wall, with one particularly dense cluster of cells expressing ELH-related mRNA along the body cavity close to the head." From this description, it cannot be determined if presumptive ELH-containing neurons, destined for central ganglia, are indeed located within typical ectodermal proliferative zones for these ganglia. The bag cell neurons, unlike neurons forming initial primordia of central ganglia in developing gastropods, do not begin to ingress from the ectoderm until after metamorphosis and their definitive position is outside the abdominal ganglion proper. Therefore, the observations of McAllister *et al.* (1983) do not contradict the notion that primordia of CNS ganglia arise initially from stereotypical ectodermal locations on the veliger body.

Pre-trochal cephalopedal ganglia

Derivation of cerebral ganglia from pre-trochal (intravelar) ectoderm, specifically the cephalic plates, was first described in early cell lineage studies of gastropods (see Conklin, 1897) and has been confirmed by many subsequent analyses (reviewed by Raven, 1958; Moor, 1983; Verdonk and van den Biggelaar, 1983). As suggested by Thompson (1958), invagination of the cephalic plates during cerebral gangliogenesis may fulfill a need for increased ectodermal surface area during rapid mitoses of cephalic plate cells.

Tardy (1970, 1974) has stated that the invaginated cephalic plates in the nudibranch *Aeolidiella alderi* are the source of both cerebral and pleural neurons. According to his interpretation, the ganglia formed from the two cephalic plates are fused cerebropleural ganglia, each having a dorsal lobe corresponding to the pleural component and a ventral lobe corresponding to the cerebral component. In young *Melibe* larvae, each developing cerebral ganglion also has a small dorsal protuberance, but these develop into the optic ganglia. The pleural ganglia of *Melibe* develop from a pair of post-trochal ectodermal placodes, as described in the following paper.

Presumptive neurons within the cephalic plate ectoderm of *Melibe* ingress singly or in small clusters throughout the larval phase; they do not separate en masse from the ectoderm during later development as suggested by Tardy (1970; 'telencephalization') for the nudibranch *Aeolidiella alderi*.

Visceral loop and osphradial neurons

The fiber tract of the visceral loop forms a complete circuit beneath the esophagus from the time *Melibe* larvae hatch from the egg mass. This fiber tract is probably established by axons from cerebral neurons, because many differentiated cerebral neurons are present at the hatching stage and the cerebral neuropiles are continuous with the visceral loop fiber tract. With the exception of several osphradial neurons and one neuron associated with the right pallial placode, differentiated neurons are not associated with the visceral loop at the hatching stage. A visceral loop arising directly from the cerebral ganglia is found in Caudofoveata, Solenogastres, Monoplacophora, and Polyplacophora, and may be the ancestral state for the molluscan nervous system (Salvini-Plawen, 1985).

Ganglia identifications

In *Melibe* larvae, all primordial ganglia associated with the visceral loop originate from visceropallial ectoderm and show some degree of torsional displacement. In prosobranchs, these characteristics apply to the intestinal and visceral ganglia, with the osphradium and osphradial ganglion also arising from visceropallial ectoderm. The pleural ganglia of prosobranchs ingress from post-trochal cephalopedal ectoderm and do not show torsional displacement (Smith, 1935; Crofts, 1937; Guyomarc'H-Cousin, 1974; Honegger, 1974; Demian and Yousif, 1975). Moritz (1939) is alone in describing a slight asymmetrical positioning for pleural ganglia in *Crepidula adunca*. I must

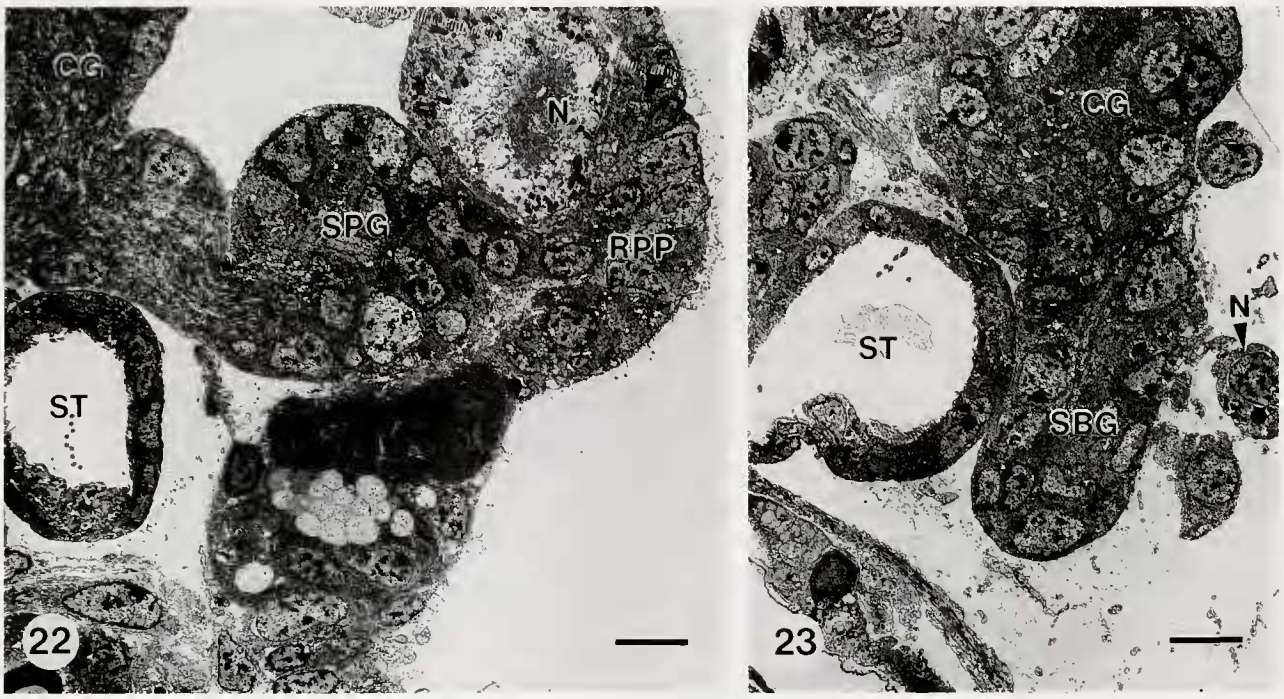


Figure 22. Longitudinal section through a larva after completing mantle retraction showing supraintestinal ganglion (SPG) projecting dorsally from right cerebral ganglion (CG). N = nephrocyst; RPP = right pallial placode; ST = statocyst. Scale, 5 μm.

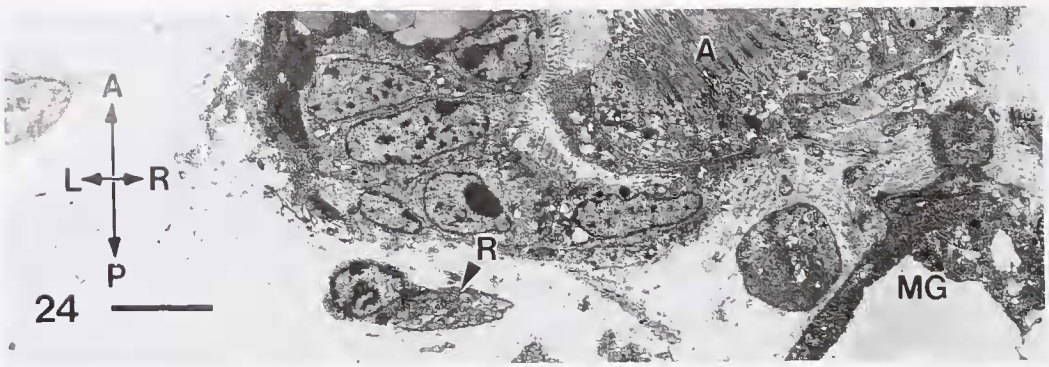
Figure 23. Longitudinal section through same larva showing concentration of subintestinal neurons (SBG) projecting ventrally from left cerebral ganglion (CG). N = ciliated tube of nephrocyst; ST = statocyst. Scale, 5 μm.

conclude that pleural neurons are not associated with the visceral loop of *Melibe* and, therefore, are not part of the adult 'cerebropleural' ganglia.

A pentaganglionate visceral loop (not including the pleural ganglia), consisting of paired parietal ganglia in addition to paired intestinal and unpaired visceral ganglia, has been called a major synapomorphy of opisthobranchs and pulmonates (Haszprunar, 1985b, 1988), even though five distinct visceral loop ganglia are rarely found among adults of this group. It is assumed that ganglionic fusions have masked the pentaganglionate condition in most Euthyneura. In support of this, Tardy (1970, 1974) and Régondaud (1961, 1964) claimed to resolve five visceral loop ganglia in a transient developmental stage of a nudibranch opisthobranch and basommatophoran pulmonate, respectively. However, the visceral loop of developing euthyneurans is short and, in *Melibe* at least, passes through a prolonged stage in which cells are distributed along the entire left limb. I argue that individual CNS ganglia cannot be defined solely by the criterion of an apparent local concentration of neuronal cells, but each must have a distinct site of neuronal ingression from the ectoderm. Only in this way can homologous ganglia be recognized in developmental stages of different gastropods. Using this criterion, which usually requires ultrastructural examination, I found only four ganglionic ingression sites along the visceral loop of *Melibe* larvae: subintestinal neurons arise from the left pallial placode; visceral neurons arise from the visceral placode; and two neuronal ingression sites were resolved for the right pallial placode, one of which must be the source of supraintestinal neurons (see below).

The neurons that differentiate from the right mantle fold ectoderm, close to its shell-secreting periphery, can be identified as osphradial by their position and because they are linked to the right limb of the visceral loop by a nerve tract. There are many descriptions of an osphradium or osphradial ganglion in larvae of prosobranchs (Thiriot-Quiévreux, 1974; Demian and Yousif, 1975; others reviewed by Fretter and Graham, 1962) and opisthobranchs that retain a mantle cavity through metamorphosis (Smith, 1967; Kriegstein, 1977a, b). However, osphradial neurons have not been identified previously in nudibranch larvae, although Kempf *et al.* (1987) found a neuron close to the edge of the right mantle fold in the nudibranch, *Tritonia diomedea,* that labelled with a monoclonal antibody to small cardioactive peptide B.

From the time of hatching, the osphradial neurons of *Melibe* are associated with a mucous cell, which suggests that the structure is homologous to the osphradial sensory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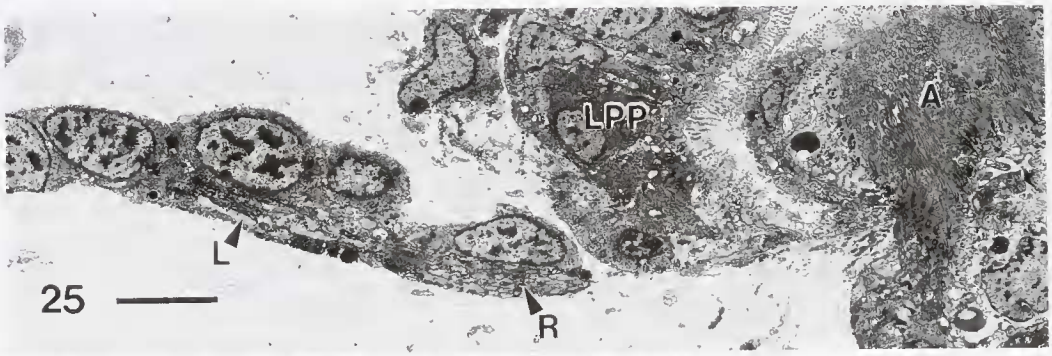
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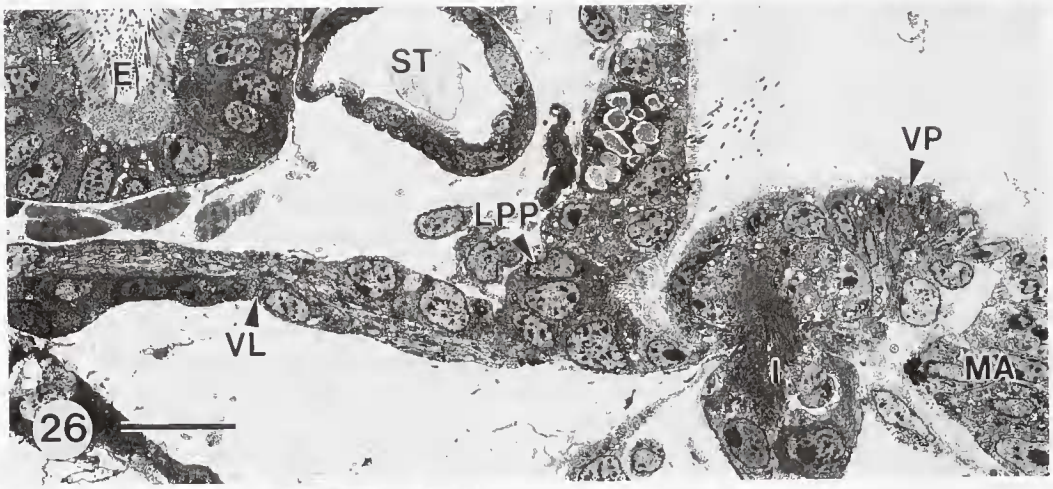
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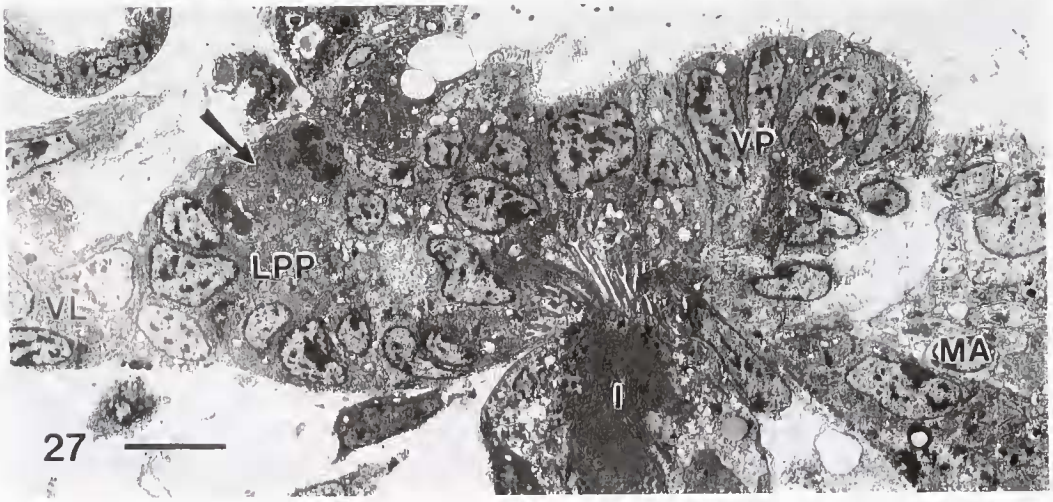
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epithelium of other gastropods. If this is the case, then is there an osphradial ganglion? Present data are insufficient to answer this question, but two possibilities can be suggested. First, if the osphradial ganglion of gastropods is generated from neurons that ingress from the osphradial sensory epithelium, as proposed by Demian and Yousif (1985), then this process does not occur during the ontogeny of *Melibe* and there is no osphradial ganglion. Consequently, the two ingression sites within the right pallial placode can be interpreted as the source of right parietal and supraintestinal neurons, respectively. Alternatively, these two ingression sites may generate neurons homologous to those of the supraintestinal and osphradial ganglia in other gastropods, in which case there is no evidence of a right parietal ganglion. The latter possibility may seem unlikely, and yet patelloid limpets (archeogastropods) have a streak of sensory epithelium along the distal mantle skirt that is connected by a nerve to a much more proximally located osphradial ganglion with overlying osphradium (Haszprunar, 1985a). Comparative developmental studies on opisthobranchs having both osphradium and osphradial ganglion could help resolve this uncertainty.

It might be argued that the more anterior of the two neuronal ingression sites within the right pallial placode is better identified as the source of right pleural neurons. However, this interpretation would require that neurons of the left pleural ganglion arise from the left pallial placode, which is obviously in a post-torsional location on the right, ventro-lateral side of the larva. No other ectodermal placode is associated with the left limb of the visceral loop in any larval stage that I examined. Furthermore, neurons that accumulate behind the left cerebral ganglion project ventrally, reflecting their post-torsional heritage. Therefore, the alternative interpretation is unacceptable because pleural ganglia are not affected by torsion in gastropods (see Fretter and Graham, 1962; Bullock, 1965). It follows that pleural ganglia must be derived from ectoderm of the cephalopedal mass and their morphogenesis is described in the companion paper.

Asymmetry of the visceral loop

The larval digestive tract of *Melibe* is torted by less than 90°, so partial torsion of visceropallial neurogenic placodes is expected. However, the visceropallial placodes exhibit differing amounts of torsion. The left pallial placode, which generates subintestinal neurons, is actually located on the right side in a position showing marked torsional displacement; it is far removed from the left nephrocyst. By contrast, torsional displacement of the right pallial placode (and the osphradial neurons) is minimal, particularly in young veligers. This placode is located immediately adjacent to the right nephrocyst. The visceral placode, like the digestive tract, exhibits approximately 60° of torsion. Consequently, all sites of pallial neurogenic ectoderm are located toward the right side of the larva, and the visceral loop is asymmetrical but never actually streptoneurous. A similar pattern of non-uniform torsion of the visceral loop was described by Crofts (1937, p. 250) for larvae of the archaeogastropod *Haliotis tuberculata*, following the initial (90°) phase of torsional twisting. Régondaud (1961, 1964) found that the visceral loop of *Lymnaea stagnalis* (Pulmonata) is only partially torted and its asymmetrical trajectory is comparable to that of *Melibe* larvae.

Cephalization and detorsion

Cephalization in gastropods is the anterior concentration of ganglia, particularly those of the visceral loop. Euthyneury is the uncrossing of the twisted visceral loop and displacement of ganglia to their pre-torsional sides; it is essentially a reversal of torsion as it affects the nervous system. Cephalization and euthyneury are believed to be prominent trends among opisthobranch and pulmonate gastropods (reviewed by Bullock, 1965; Schmekel, 1985).

Naef (1911) appears to have originated the idea that euthyneury is due to shortening of the visceral loop. He suggested that shortening concentrates the ganglia in the head, thereby pulling them out of the body area subject to torsion. This notion was more recently reiterated by Tardy (1970). Naef's theory is false because observations

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Figures 24–27. Series of frontal sections through larva at onset of mantle retraction showing merger of right and left limbs of visceral loop and left pallial and visceral placodes. Orientation arrows: A = anterior; P = posterior; L = left; R = right.

Figure 24. Right limb of visceral loop (R) extending toward left from mantle gland (MG). A = anus. Scale, 5 μm.

Figure 25. Merger of right and left limbs of visceral loop (R and L, respectively) adjacent to anus (A) and left pallial placode (LPP). Scale, 5 μm.

Figure 26. Left limb of visceral loop (VL) associated with left pallial placode (LPP). Section grazes through periphery of visceral placode (VP) adjacent to terminal intestine (I). E = esophagus; MA = mantle; ST = statocyst. Scale, 10 μm.

Figure 27. Left pallial placode (LPP) with mitotic cell (arrow) and visceral placode (VP). I = intestine; MA = mantle; VL = visceral loop. Scale, 5 μm.

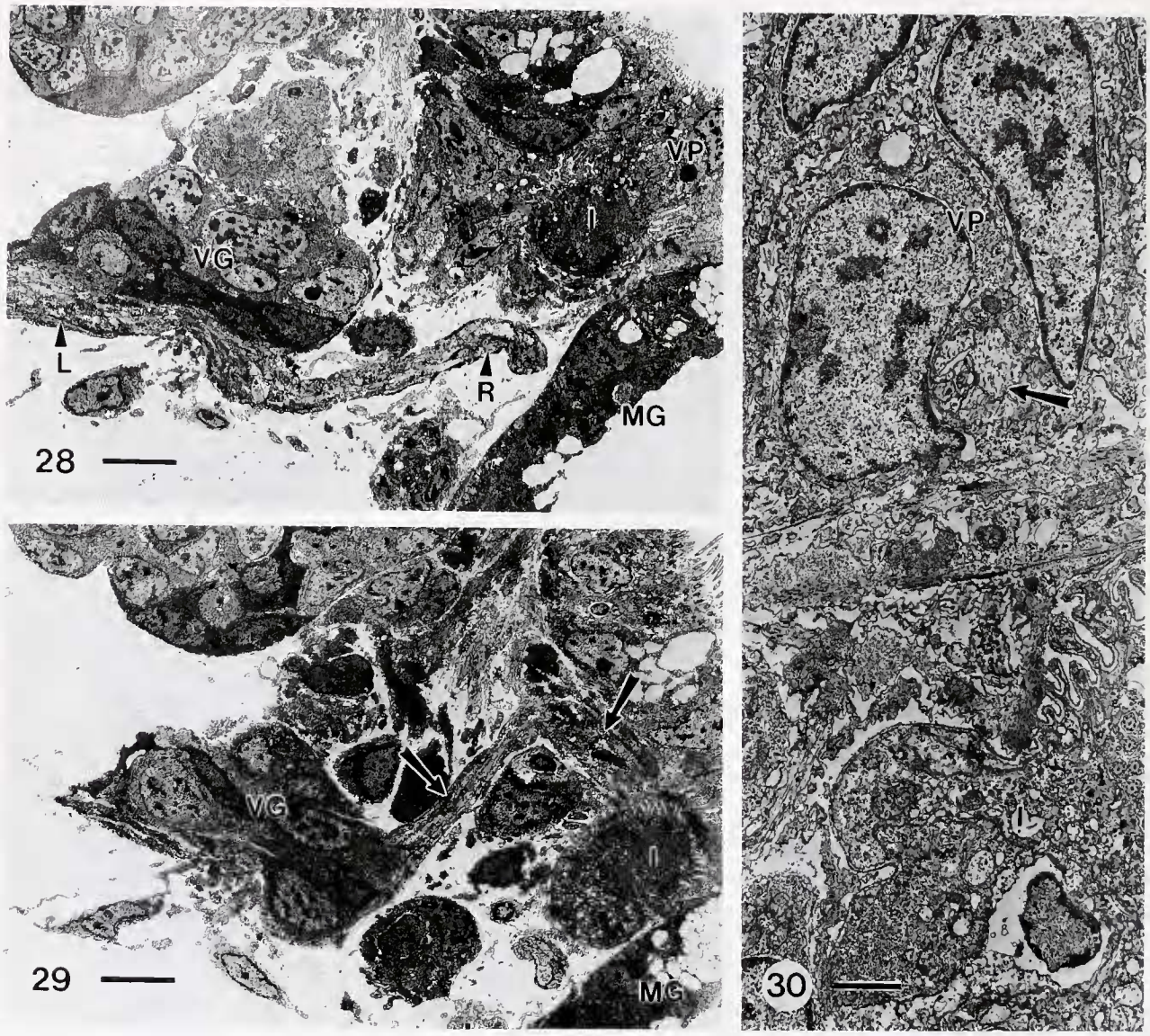


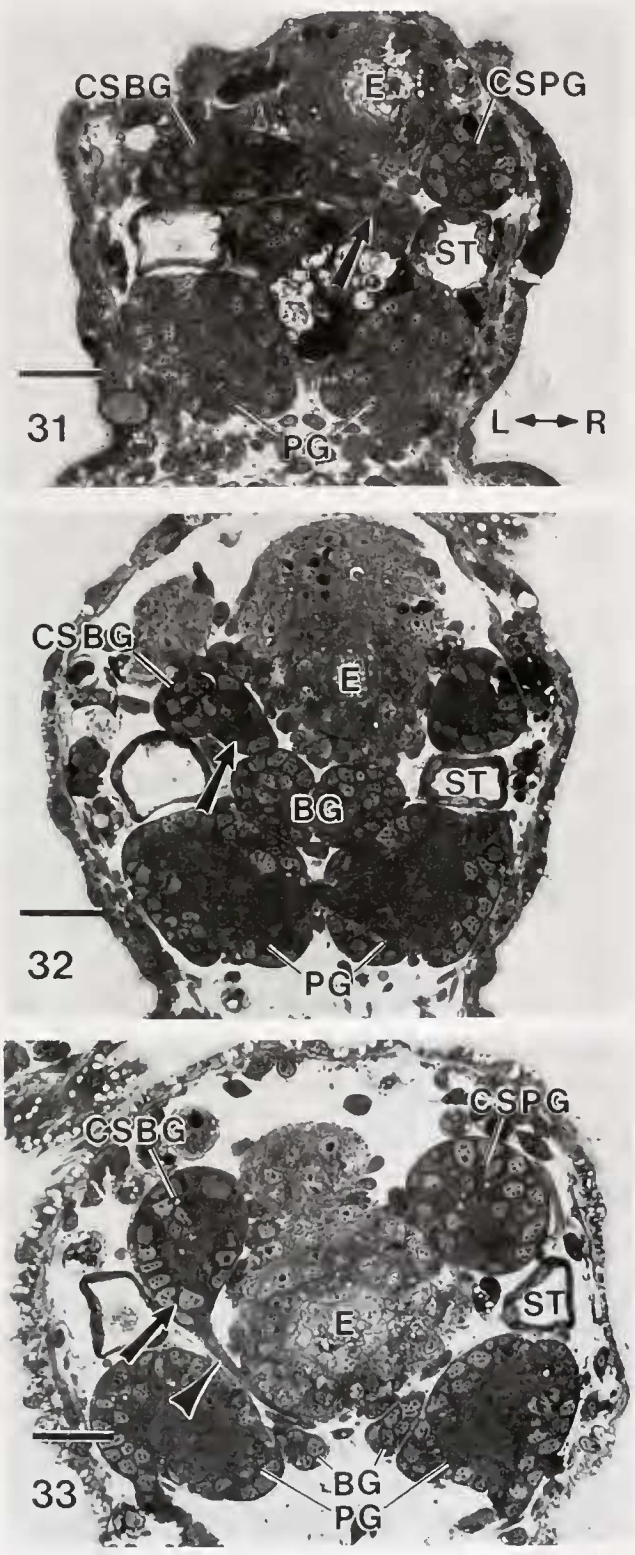
Figure 28. Frontal section through larva at mantle fold hypertrophy stage showing merger of right and left limbs of visceral loop (R and L, respectively) adjacent to intestine (I). MG = mantle gland; VG = visceral ganglion; VP = visceral placode. Scale, 5 μm.

Figure 29. Slightly deeper section showing visceral nerve (arrows) extending around distal intestine (I) from visceral ganglion (VG) towards visceral placode. Scale, 5 μm.

Figure 30. Visceral nerve (arrow) within base of visceral placode (VP). I = intestine. Scale, 1 μm.

on *Melibe* and *Lymnaea stagnalis* (Régondaud, 1961, 1964) show that visceral loop ganglia differentiate from torted visceropallial ectoderm, as they do in prosobranchs. Nevertheless, the concept of visceral loop shortening as the cause of euthyneury is popular (see Fretter and Graham, 1962; Bulloch, 1965). It might be envisioned that visceral loop ganglia become concentrated against the cerebral ganglia of their respective pre-torsional sides as threaded beads pushed together during shortening of a convoluted string. However, as pointed out previously by Régondaud (1961) for *Lymnaea*, and also seen in *Melibe* and *Aplysia californica* (Kriegstein, 1977a, b), all visceral loop ganglia differentiate within close proximity along a short visceral loop. This loop simply remains short in cephalized species, while the rest of the body elongates after metamorphosis. Instructions to elongate are given instead to peripheral nerves. This is more than a semantic point if cephalization is to provide a mechanism for eu-

thyneury, because visceral loop ganglia of *Melibe* and *Lymnaea* are close together *and* torted at the outset of gangliogenesis.



I propose that euthyneury in *Melibe* can be explained by two factors. First, half of the hypothesized detorsion is fictional because ganglia differentiate from visceropallial ectoderm that never shows more than partial torsion. Second, existing evidence suggests that subintestinal neurons, which arise from visceropallial ectoderm showing marked torsional displacement (unlike supraintestinal neurons), move toward the left cerebral ganglion by active migration along the visceral loop fiber tract. Other evidence that neurons migrate along existing connectives during gastropod neurogenesis comes from the study of McAllister *et al.* (1983), who found evidence that bag cells migrate along the visceral loop in juveniles of *Aplysia californica*. Active, leftward migration of subintestinal neurons contributes to both cephalization and detorsion without involving a length change of the visceral loop.

Similarly, visceral neurons appear to migrate along the nerve linking visceral placode and visceral loop during later larval development. However, it is unlikely that active migration of neurons is responsible for the final displacement of the visceral ganglion to the left side during metamorphosis, because the movement is rapid and involves all visceral neurons simultaneously. Instead, metamorphic movement of the visceral ganglion may be accomplished by differential lengthening of various connectives and commissures linking the CNS ganglia. Lengthening is restricted to the pedal and parapedal commissures and the segment of visceral loop extending between the supraintestinal and visceral ganglia (elongation of the latter is still minimal compared to that which occurs after metamorphosis in *Aplysia californica*). Therefore, when the larval body expands rapidly after shell loss, the visceral ganglion is pulled to the left because its tether to the left side of the CNS remains short, whereas that connecting it to the right side lengthens. Displacement of the visceral ganglion to the left side 'overshoots' detorsion.

Conclusions

Results of this study suggest that the paired 'cerebropleural' ganglia of *Melibe leonina* are, in reality, a fusion

Figures 31–33. Histological cross sections through metamorphic stages showing movement of visceral ganglion (arrow) from right to left sides of post-larva. Orientation arrows: L = left; R = right. BG = buccal ganglia; E = esophagus; CSBG = left cerebral + subintestinal ganglia; CSPG = right cerebral + supraintestinal ganglia; PG = pedal ganglia; ST = statocyst. Scales, 25 μm.

Figure 31. Immediately after shell loss.

Figure 32. Five hours after shell loss.

Figure 33. Ten hours after shell loss. Visceral ganglion has fused with left cerebral + subintestinal ganglionic complex. Arrowhead indicates visceral loop fiber tract.

of cerebral, subintestinal, and visceral ganglia on the left side, and cerebral, supraintestinal, and possibly parietal and osphradial ganglia on the right side.

The basic configuration of the post-metamorphic CNS and the pattern of peripheral nerves among many nondorid nudibranchs are generally similar to that of *Melibe leonina*. Among dendronotaceans, Dorsett (1978) extended this to include similarities at the level of individual neurons, despite differences in adult body form. Therefore, my conclusions about the identity and fate of visceral loop ganglia may apply to many other non-dorid nudibranchs. However, small differences in adult neuroanatomy may signify large morphogenetic differences. Even among members of the genus *Melibe*, Gosliner (1987) has documented differences in relative size and surface texture of CNS ganglia. Development of visceral loop ganglia in aeolids such as *Phestilla sibogae*, in which the dorsum and cerata are derived from epipodial ectoderm rather than pallial ectoderm (Bonar and Hadfield, 1974; Bonar, 1976), may be quite different from that in *Melibe*.

Dorid nudibranchs form a cohesive group with features that distinguish them from other nudibranchs (Minichev, 1970; Schmekel, 1985), including anatomical details of the CNS and pattern of peripheral nerves. Ganglionic regions in the CNS of dorids requires separate neurodevelopmental analysis.

Acknowledgments

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